Determining Viable Microbiome in Anaerobic Treatment Processes by Propidium Monoazide (PMA)-PCR

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学位論文題目	Determining Viable Microbiome in Anaerobic Treatment Processes by Propidium Monoazide (PMA)-PCR （PMA-PCR 法を用いた嫌気性処理プロセスにおける"生きている"微生物の同定と定量）
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論文内容要約

Anaerobic digestion technology is one of the most important biomass utilization technologies. It converts solid organic matters into dissolved organic matters, and then converts the energy stored in waste into biogas for combustion or power generation, so as to realize the resource and energy recovery; less residual amount after anaerobic digestion; closed reaction equipment, can control the emission of malodor. Anaerobic treatment greatly improves the energy balance of the organic waste treatment process, and has great economic and environmental advantages. In view of the fact that anaerobic digestion (AD) system is a biochemical reaction process dominated by microorganisms, uncovering the microbial community can help optimize system performance and ensure process stability. Since the anaerobic treatment processes was formed as hydrolysis step, acidogenesis step, acetogenesis and methanogenesis step, microbes participating during those steps were classified as hydrolyzing and fermenting microorganisms, acidogenic bacteria, methanogens and other microorganisms. In recent years, many studies have used 16S rRNA gene sequencing technology to explore the composition of microbial communities in AD systems.

However, some aerobic microorganisms were also detected in anaerobic samples according to previous studies, especially for samples taken from full-scale anaerobic treatment processes. One major reason is that the residue of legacy DNA extracellularly in environments and DNA in decaying cells staying for a long time causes the bias of the results. It is also necessary to know the composition of the microorganisms domesticated in anaerobic treatment processes.

Two typical anaerobic digestion technology are necessary to be asked to investigate viable microbial community structure: the anaerobic sludge digester and the anaerobic membrane bio-reactors (AnMBRs) for treating sewage sludge and sewage, respectively. There are two major reasons: 1) the substrate contains a large number of microorganisms, which will interfere with the determination of viable / contributed microorganisms; 2) long sludge retention time (SRT) slows the discharge of residual cells, which can lead to bias in the detection of viable microbial communities. For AnMBRs, there is another reason, i.e., the exitance of membrane hold on almost of the microorganisms.

So far, some efficient methods have been developed to eliminate the effects from those dead cells, and each of them has merits and demerits. One of the methods was based on the cell membrane integrity which has been wildly used for many years. This method can successfully help the eliminated DNAs extracted from dead cells with compromised cell membrane. propidium

monoazide (PMA)-PCR is one of the membrane integrity-based approach which can analysis DNAs from viable cells with intact cell membrane. PMA is one of the photoreactive DNA-binding dye that preferentially and only binds to DNA of cells with compromised membranes (assumed to be dead or damaged cells). And after PCR, only those DNAs extracted from intact calls can be amplified and detected by next generation sequencing (NGS). This method is commonly used in medical as well as environmental fields. The experimental operation of this method is relatively simple, and it can be combined with other microbial analysis methods, so it has been used in other ways such as pathogens' detection. In this thesis, the quantification and community structure analysis of viable microorganisms in the anaerobic digestion processes were determined using PMA-PCR.

In chapter 3, PMA-PCR with 16S rRNA gene sequencing analysis was used to distinguish live and dead microorganisms based on cell membrane integrity. Microbial community structures of PMA treated and untreated anaerobic digester sludge samples were compared. Quantitative PCR revealed that 5-30% of the rRNA genes were derived from inactive or dead cells in anaerobic sludge digesters. This caused a significant decrease in the numbers of operational taxonomic units and Chao1 and Shannon indices compared to that of the PMA untreated sludge, which indicated DNA extracted from dead cells with compromised cell membrane were eliminated by PMA treatment. Microbial community analysis showed that majority of the viable microbiome consisted of *Euryarchaeota*, *Bacteroidetes*, *Deltaproteobacteria*, *Chloroflexi*, *Firmicutes*, WWE1, *Spirochaetes*, *Synergistetes*, and *Caldiserica*. On the other hand, after the PMA treatment, numbers of *Alphaproteobacteria* and *Betaproteobacteria* declined. These were considered as residual microbial members. Major OTUs belonged to WWE1 and *Bacteroidales* maintained a certain population in each sample. The network analysis also revealed a relationship among the OTUs belonging to WWE1 and *Bacteroidales*. Two groups were classified by network analysis, and positive correlations was within the same group but negative correlation was in another group.

In chapter 4, effects of membrane pore size and HRT operational factors to microbial community's difference in anaerobic bio-membrane reactors were evaluated. Two AnMBRs with different membrane pore sizes: AnMBR_1 with 0.4 μm pore size, AnMBR_2 with 0.05 μm pore size. AnMBRs were operated under mesophilic condition, and HRT was changed from 24h to 4h and 24h to 10h, respectively. Comparation of microbial structure before and after PMA treatment was performed in order to detect viable microbiome in the reactors. Microbial diversity analysis of samples taken from the reactors and from the membrane surface revealed relationship and difference between community structures. As a result, *Anaerolineae Bacteroidia* and *Clostridia* were predominant in both two reactors. For AnMBR_1: as the HRT changed from 24h to 8h, the relative abundance of *Anaerolineae* increased and reached to a peak when under HRT 8h. However, as the HRT continued to decreased to 4h, the relative abundance of *Anaerolineae* decreased to 18.9%. However, *Bacteroidia* showed opposite tendency during the whole process. In AnMBR_2, the above two classes show similar tendency, but HRT node became 10h. *Clostridia* decreased from 34.9% to 7.3% in AnMBR_2, with the decrease of HRT, losing its relative stability while compared to the results showed

in AnMBR_1. Principal Co-ordinates Analysis (PCoA) gave the evidence that as the HRT decreased, the microbial community changed in the two reactors tend to be consistent. At HRT 4h and 10h (last period of the two systems), respectively, the microbial community structure showed a large changing, deviating from the trend of stabile periods. Major members of microbes showed a similarity on the surface of the membrane and in the reactors. OUTs close to *Petrimonas sulfuriphila* and family *Porphyromonadaceae* were specifically dominant on the surface of membrane. *Anaerolineae*, *Bacteroidia*, *Clostridia*, *Betaproteobacteria*, and *Deltaproteobacteria* were consisted of major viable microbiome in AnMBRs. PMA-PCR based 16S rRNA gene sequencing successfully determining viable microbiome in AnMBR samples.

In chapter 5, 16S rRNA gene sequencing revealed microbial community structure in an AnMBR at low temperature. Conventional AnMBR reactors were always carried out under mesophilic to thermophilic conditions in order to owning a high treatment efficient. However, in most cities, the water temperature always under 20˚C in winter, and extra cost is needed for maintaining mesophilic and thermophilic temperature. Low temperature will also affect the activity of anaerobic microorganisms. Compared with the mesophilic and thermophilic anaerobic reactors, especially for those treating municipal sewage, dead microorganisms would be more difficult to be degraded, which will affect the analysis of microbial diversity. This part of the research was aimed to determine a viable microbial community structure in an AnMBR reactor at low temperature (20˚C and 15˚C) by using the PMA-PCR method. As a result, qPCR showed that 17.3% less gene copy numbers in samples taken at 15˚C after PMA treatment in average which could be considered from dead cells. The difference of alpha-diversity with and without PMA treatment implied that in the AnMBR reactor near 16% OTUs could be considered as dead cells. In order level, *Bacteroidales* were the most abundant groups, followed by *Clostridiales*, *Anaerolineales*, *Lactobacillales* and *Synergistales* in relative abundance without PMA treatment. With PMA treatment, *Firmicute* and *Chloroflexi* showed more obvious decrease at lower temperature (15˚C) than at 20˚C in samples, and as the HRT increased from 6h to 24h, their difference became smaller. The obvious reduction of gene copy numbers of major orders indicated that most members of *Clostridiales Anaerolineales* and *Lactobacillales* can be considered as dead cells. *Campylobacterales*, *Rhodocyclales*, and *Ignavibacteriales* only showed a little reduction or even increased after PMA treatment, which can be considered as viable populations in psychrophilic AnMBR. Viable microbial members in OTU level after PMA treatment showed that majority of viable microorganisms belonged to *Euryarchaeota*, *Bacteroidetes*, *Proteobacteria*, *Synergistetes*, *Firmicutes*, *Chlorobi* and *Spirochaetes*.

In chapter 6, the results and discussion were summarized. This PMA-PCR treatment combining with qPCR and NGS methods was proved as one of the simple and efficient methods determining the viable microbiomes in anaerobic treatment processes. The biases caused by residual DNAs of aerobic microorganisms can be successfully excluded by the PMA-PCR method. Lots of previous studies chose PMA treatment to distinguish dead and viable cells. Most of them only use qPCR to quantify the amount of the total viable microbiomes, and therefore, it lacks the information about the abundance of each microorganisms.

Several studies used NGS to detect the relative abundance of viable microbiome; however, the results did not show the absolute numbers of microorganisms. The most important point of this research is to explore if it is possible to use the same primer set for qPCR and NGS analysis combined with PMA treatment. The results indicated that this attempt is undoubtedly successful, and in the future, this simple method can also be applied to other samples in other fields, not limited to anaerobic samples, only using the same primer set for uncovering viable microbiome in complex environmental samples.

The ideas of this research can also be applied to the currently popular metagenomic approach, in order to detect the viable microbial population structure, evolutionary relationships, functional activities and the cooperative relationship between the environment.